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Title

Universal screening for Lynch Syndrome in a large consecutive cohort of Chinese colorectal cancer patients: high prevalence and unique molecular features

Author

Wu Jiang^{1,2}, Muyan Cai^{1,3}, Shiyong Li⁴, Jinxin Bei^{1,5}, Fang Wang^{1,6}, Heather Hampel⁷, Yihong Ling^{1,3}, Ian M. Frayling^{8,9}, Frank A. Sinicrope¹⁰, Miguel A. Rodriguez-Bigas¹¹, James J. Dignam¹², David J. Kerr¹³, Rafael Rosell¹⁴, Mao Mao⁴, Jibin Li¹⁵, Yunmiao Guo^{1,5}, Xiaoyan Wu^{1,6}, Lingheng Kong^{1,2}, Jinghua Tang^{1,2}, Xiaodan Wu^{1,2}, Chaofeng Li¹⁶, Jierong Chen^{1,5}, Qingjian Ou^{1,2,5}, Mingzhi Ye⁴, Fengming Guo⁴, Peng Han⁴, Qiwei Wang¹⁷, Desen Wan^{1,2}, Li Li¹⁸, Ruihua Xu^{1,19}, Zhizhong Pan^{1,2}, Peirong Ding^{1,2}

Written on behalf of AME Colorectal Cancer Cooperative Group

Author Affiliations:

1. Sun Yat-sen University Cancer Center; State Key Laboratory of Oncology in South China; Collaborative Innovation Center for Cancer Medicine, Guangzhou 510060, China
2. Department of Colorectal Surgery, Sun Yat-sen University Cancer Center, Guangzhou, P.R. China.
3. Department of Pathology, Sun Yat-sen University Cancer Center, Guangzhou, P.R. China.

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4. BGI-Guangzhou Medical Laboratory, BGI-Shenzhen, PR China.
5. Department of Experimental Research, Sun Yat-sen University Cancer Center, Guangzhou, P.R. China.
6. Department of Molecular Diagnostics, Sun Yat-sen University Cancer Center, Guangzhou, P.R. China.
7. Division of Human Genetics, Department of Internal Medicine, The Ohio State University Comprehensive Cancer Center, Columbus, OH, USA.
8. Institute of Cancer and Genetics, Cardiff University, Cardiff, UK.
9. All-Wales Medical Genetics Service, Institute of Medical Genetics, University Hospital of Wales, Cardiff, UK.
10. Department of Oncology, Mayo Clinic, Rochester, Minnesota
11. Department of Surgical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas
12. Department of Public Health Sciences, The University of Chicago Biological Sciences, Chicago
13. Department of Clinical Pharmacology, OCRB, Churchill Campus, Headington, University of Oxford, Oxford, UK
14. Catalan Institute of Oncology, Germans Trias i Pujol Health Sciences Institute and Hospital, Badalona, Barcelona
15. Department of Clinical Research, Sun Yat-sen University Cancer Center, Guangzhou, P.R. China
16. Department of Information Technology, Sun Yat-sen University Cancer Center,

Guangzhou, P.R. China.

17. State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, P.R. China.

18. Imaging Diagnosis and Interventional Center, Sun Yat-sen University Cancer Center, Guangzhou, P.R. China

19. Department of Medical Oncology, Sun Yat-sen University Cancer Center, Guangzhou, P.R. China.

Wu Jiang, Muyan Cai, Shiyong Li and Jinxin Bei contributed equally to this article.

Correspondence to:

Prof. Peirong Ding, Department of Colorectal Surgery, Sun Yat-sen University Cancer Center, 651 Dongfeng East Road, Guangzhou 510060, People's Republic of China; Tel: +86 20 87343920, E-mail: dingpr@sysucc.org.cn

Prof. Ruihua Xu, Department of Medical Oncology, Sun Yat-sen University Cancer Center, 651 Dongfeng East Road, Guangzhou 510060, People's Republic of China; E-mail: xurh@sysucc.org.cn,

Prof. Zhizhong Pan, Department of Colorectal Surgery, Sun Yat-sen University Cancer Center, 651 Dongfeng East Road, Guangzhou 510060, People's Republic of China; E-mail: panzhzh@sysucc.org.cn

Running head

Universal screening for LS among Chinese CRC patients

Key words: Colorectal cancer; Lynch Syndrome; universal screening; ethnic diversity

Abbreviations

LS, Lynch syndrome; MMR, mismatch repair; CRC, colorectal cancer; dMMR, MMR protein deficiency; IHC, immunohistochemical; PCR, polymerase chain reaction; CNV, copy number variations; InSiGHT, International Society of Gastrointestinal Hereditary Tumors; LOVD, Leiden Open Variation Database; VUS, variants of uncertain significance

Novelty & Impact Statements

The current study demonstrated that there are unique molecular features in a large consecutive Chinese colorectal cancer cohort undergoing universal screening for Lynch Syndrome, characterized by high prevalence and infrequent *BRAF*^{V600E} mutation. These results verified the ethnic diversity among lynch syndrome. Patients older than 65 years who do not meet the revised Bethesda guidelines have a low risk of lynch syndrome, suggesting germline sequencing might not be necessary in this population.

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Conflicts of Interest

Ms Hampel discloses a scientific advising role with Invitae and Genome Medical, research collaborations with Ambry, Invite, and Myriad Genetics Inc, and stock in Genome medical. No other conflicts are reported.

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ABSTRACT

The prevalence of Lynch syndrome (LS) varies significantly in different populations, suggesting that ethnic features might play an important role. We enrolled 3330 consecutive Chinese patients who had surgical resection for newly diagnosed colorectal cancer. Universal screening for LS was implemented, including immunohistochemistry for mismatch repair (MMR) proteins, *BRAF*^{V600E} mutation test and germline sequencing. Among the 3250 eligible patients, MMR protein deficiency (dMMR) was detected in 330 (10.2%) patients. Ninety-three patients (2.9%) were diagnosed with LS. Nine (9.7%) patients with LS fulfilled Amsterdam criteria II and 76 (81.7%) met the revised Bethesda guidelines. Only 15 (9.7%) patients with absence of MLH1 on IHC had *BRAF*^{V600E} mutation. One third (33/99) of the MMR gene mutations have not been reported previously. The age of onset indicates risk of LS in patients with dMMR tumors. For patients older than 65 years, only 2 patients (5.7%) fulfilling revised Bethesda guidelines were diagnosed with LS. Selective sequencing of all cases with dMMR diagnosed at or below age 65 years and only of those dMMR cases older than 65 years who fulfill revised Bethesda guidelines results in 8.2% fewer cases requiring germline testing without missing any LS diagnoses. While the prevalence of LS in Chinese patients is similar to that of Western populations, the spectrum of constitutional mutations and frequency of *BRAF*^{V600E} mutation is different. Patients older than 65 years who do not meet the revised Bethesda guidelines have a low risk of LS, suggesting germline sequencing might not be necessary in this population.

Introduction

Lynch syndrome (LS), is the most common hereditary colorectal cancer (CRC) syndrome, accounting for 2%-4% of all CRC cases.¹⁻⁴ LS is caused by germline mutations in one of four mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6* and *PMS2*)⁵, or deletions in the 5' area of *EPCAM* which result in hypermethylation of the *MSH2* promoter and subsequent *MSH2* silencing.^{6, 7} Patients with LS are susceptible to various cancers and often at a young age.⁸⁻¹¹ Both the US National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology for Genetic/Familial High-Risk Assessment (Colorectal) and the National Institute for Health and Care Excellence (NICE) in the UK now recommend universal screening of all CRC to improve the identification of individuals with LS.^{12, 13} The prevalence of LS varies significantly in different populations, suggesting that ethnic diversity might play an important role in the disease. To our knowledge, screening for LS has been mainly performed in western populations and data regarding Chinese patients is scarce. Because of China's large population, delineation of the prevalence and genotype of LS in Chinese patients would help to understand the ethnic diversity of LS.

Since the universal screening strategy has been gradually adopted worldwide, it is interesting to explore the prevalence of LS in patients with MMR protein deficiency (dMMR) in different age of onset. In this way, we could identify the patients with high risk of LS for germline sequencing and optimize the screening strategy.

Herein, we conducted universal screening for LS in a consecutive large cohort with newly diagnosed CRC, using immunohistochemical (IHC) staining for MMR proteins, followed by *BRAF*^{V600E} testing in cases with absence of MLH1, and then multigene panel testing on germline DNA in all cases with dMMR and no *BRAF*^{V600E} mutation. We investigated the prevalence of dMMR and LS in these unselected CRC patients and further evaluated the associations between age of onset and prevalence of LS in patients with dMMR tumors. Finally, the efficiency of a selective strategy consisting of universal tumor MMR testing and specific clinical criteria was tested.

Materials and Methods

Patients and Screening strategy

Between November 2011 and December 2015, 3330 consecutive patients who had surgical resection for newly diagnosed colorectal adenocarcinoma at the Sun Yat-sen University Cancer Center, China were enrolled in this study. Eighty patients with a clinical diagnosis of polyposis or with insufficient tumor tissue for IHC were excluded. The protocol was approved by the clinical research ethics committee of the Cancer Center. All patients provided written informed consent to participate in the study. All data in our study have been recorded at Sun Yat-sen University Cancer Center for future reference (number RDDA2018000384).

The screening strategy is detailed in Figure 1. In brief, IHC for four MMR proteins was performed universally to identify patients with dMMR as evidenced by the

absence of one or more of the MMR proteins. If the MLH1 protein was absent, *BRAF*^{V600E} mutation testing was performed in the tumor to exclude cases with suspected *MLH1* gene promoter methylation. Patients with dMMR and no *BRAF*^{V600E} mutation were referred for genetic counseling and identified as candidates for germline sequencing.

IHC and *BRAF*^{V600E} mutation testing

All specimens were prepared as 4μm FFPE sections. The sections were tested by a validation program in the Leica-bond system (Leica Biosystems Nussloch GmbH, Germany). Primary monoclonal antibodies against MLH1 (cloneES05; ZSGB-BIO, Beijing, China), MSH2 (clone RED2; ZSGB-BIO, Beijing, China), MSH6 (clone EP49; ZSGB-BIO, Beijing, China), and PMS2 (cloneEP51; ZSGB-BIO, Beijing, China) were applied to the sections. Protein expression was assessed by two independent pathologists (CMY, LYH). Protein deficiency was defined as absence of nuclear staining within tumor cells and positive nuclear staining in normal tissues as internal control. Preserved expression was defined as nuclear staining in tumor cells with consistent labeling in control cells.

The *BRAF*^{V600E} mutation within exon 15 was detected using fluorescent real-time polymerase chain reaction (PCR). Genomic DNA was amplified in a 24μL PCR reaction with 7500 real-time fluorescence quantitative PCR system (Applied Biosystems, Foster City, CA). Mutations were confirmed with independent duplicate

analyses.

Germline DNA sequencing and Variant classification

DNA was extracted from peripheral blood samples by QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's standard protocol. NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was used to assess the DNA quality and concentration. Qualified DNA samples were used to do the library construction. The insert size and quantity of the library were assessed by Bioanalyzer 2100 (Agilent Technologies, Santa Clara, MA, USA) instrument and enrichment of the target region was assayed by qPCR. After quality control, the library was sequenced by a multigene panel using the HiSeq platform, in which the target regions of *MLH1*, *MSH2*, *MSH6*, *PMS2* and *EPCAM* were covered.

After filtering the low quality reads, clean sequencing data were aligned to reference (UCSC, hg19) using BWA0.6.2 (Burrow-wheeler Aligner), marked for duplication by Picard (V1.98) and re-aligned by GATK (v3.5, Broad Institute, Cambridge, MA, USA). After that, the final mapping file (BAM format) was used to detect the SNP/InDel by GATK software. In-house developed software was used to detect gene exonic deletion/duplication mutation. SNP/InDel mutations with less than 25% mutational frequency were filtered. Mutations were annotated based on RefSeq GTF file. The SNP frequency was annotated by Dbsnp141, ExAC, 1000 genomes project and so on. The pathogenicity of mutations was classified according to American

College of Medical Genetics and Genomics (ACMG) recommendations.¹⁴ All the pathogenic and likely pathogenic SNP/InDel mutations were confirmed by Sanger sequencing. All the copy number variations (CNV) were validated by qPCR method.

Domain region in the MMR genes was defined by Pfam database. We took the known pathogenic missense mutations from our in-house database as positive control and the in-house benign missense database and mutations with >0.01 allele frequency in human published databases served as a negative control. If one domain region only contains pathogenic mutation, it was defined as a highly conserved functional domain. Those mutations located in highly conserved regions were considered as moderate evidence for pathogenic nature. Any missense mutations of uncertain significance were re-evaluated for their location, in silico prediction results, phenotype, family history, and also checked against the International Society of Gastrointestinal Hereditary Tumors (InSiGHT) reference Leiden Open Variation Database (LOVD) at <http://www.insight-database.org/genes> in order to further assess pathogenicity.

Statistical Analysis

Demographic data were summarized as mean (\pm standard deviation) or frequency (%). Sensitivity, Specificity, positive predictive value, negative predictive value and diagnostic yield of screening strategies were calculated. SPSS statistical software, version 17(Chicago, USA: SPSS Inc.) were used to perform the analyses.

Results

Prescreening for LS

A total of 3250 patients were eligible for analysis. Men accounted for 59.8% (n = 1942) of patients. The mean age at CRC diagnosis was 57.3 years (range, 15 to 91 years). Abnormal IHC results were detected in 330 (10.2%) CRC tumors, including: 158 patients (47.9%) with MLH1 loss alone or with loss of PMS2, 98 patients (29.7%) with absent MSH2 with or without MSH6 loss, 26 patients (7.9%) with only MSH6 absent, 26 patients (7.9%) with only PMS2 absent, and 22 patients (6.7%) with other combinations (e.g. loss of 3 MMR proteins or unpaired loss). Demographic and clinical characteristics for the 330 patients with dMMR tumors are listed in Table 1.

In 154 patients whose tumors had loss of MLH1, *BRAF*^{V600E} mutation testing was performed. Only 15 (9.7%) patients carried *BRAF*^{V600E} mutation which is consistent with a sporadic origin. Thus, the remaining 315 patients with abnormal IHC and no *BRAF*^{V600E} mutation were referred for genetic counseling. Of these, 256 (81.3%) agreed to undergo germline sequencing, while the other 59 patients refused further testing.

Outcomes of Germline sequencing

Among the 256 patients with dMMR tumors who underwent germline genetic testing, 99 pathogenic or likely pathogenic mutations were discovered in 93 patients (eTable 1 in the Supplement). Therefore, the prevalence of Lynch syndrome is 2.9% (93/3191)

in our cohort. Germline mutations in *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM* accounted for 42.4%, 36.4%, 15.2%, 5.1%, and 1.0% of all mutations, respectively. One third (33/99) of the mutations have not been reported previously. The c.793C>T missense mutation in *MLH1* (7 patients) and c.1452_1455 deletion mutation in *MSH2* (6 patients) were the two most common mutations. Overall, we found 35 frameshift mutations, 25 nonsense mutations, 14 missense mutations, 14 CNVs, 10 splice site mutations and 1 in-frameshift mutation. Frameshift mutations were the most common type found in *MLH1*, *MSH2* and *MSH6*, while pathogenic missense mutations were common in *MLH1* (eFigure in the Supplement).

There were 70 variants of uncertain significance (VUS) in MMR genes that were detected in 57 patients. Missense mutations were the most common type and accounted for 64.3% of the VUS. Twenty-seven (60%) of the missense mutations were consistent with IHC results, showing loss of the protein product of the mutant gene (eTable 2 in the Supplement). Four variants fulfilled criteria for upgrading to likely pathogenic. Given that the conserved functional domain without benign variation was established based on an in-house database, we classified these 4 patients as highly suspicious for having LS and therefore, close clinical follow up was recommended.

Strategies for Screening

Clinical strategies for LS screening were also evaluated (Table 2). Only 9 (9.7%)

patients with LS fulfilled Amsterdam criteria II. In addition, using the revised Bethesda guidelines as a prescreening method would have left 17 (18.3%) cases undiagnosed. Although the universal tumor screening strategy provides the greatest sensitivity, its positive predictive value was relatively low at 36.3%. We also evaluated the relationship between age of onset and prevalence of LS in patients with dMMR tumors: 15 of the 41 patients (36.6%) \leq 40 years, 39 of 71 patients (54.9%) aged 41 – 50 years, 28 of 80 patients (35.0%) aged 51 – 60 years, 9 of 29 patients (31%) aged 61 – 65 years, 1 of 15 patients (6.7%) aged 66–70 years, and 1 of 20 patients (5.0%) aged 71 years or older (Figure 2). Considering the indispensable role of tumor MMR status in clinical practice and the low frequency of LS in patients older than 65 years in our cohort, a selective strategy consisting of universal tumor MMR testing and specific clinical criteria was tested. When germline sequencing was only performed in patients with dMMR tumors who were diagnosed at 65 years or younger, and in older patients fulfilling at least 1 criterion of the revised Bethesda guidelines, 8.2% fewer cases would be candidates for germline sequencing with none of the LS patients being missed and positive predictive value modestly improved.(Table 2)

Discussion

Previous studies have shown that universal screening of all CRC cases for LS by analysis of microsatellite instability or IHC for MMR proteins is the most sensitive¹⁵ and probably most cost-effective strategy.¹⁶⁻¹⁸ However, its application in Chinese population has not been well investigated, especially in a large unselected CRC series.

Furthermore, there were 376,300 new diagnoses of CRC in 2015 in China¹⁹, nearly one fourth of the new cases worldwide. Although our patients were mainly from southern China, the results still could be a great supplementary to the ethnic diversity of LS and fundamental data for direct implications on clinical practice.

In our study, 330 (10.2%) patients were found to have tumors with dMMR and 93 (2.9%) were diagnosed as LS. Considering that 59 (18.7%) patients with dMMR and no *BRAF*^{V600E} mutation did not receive germline sequencing, and the possibility of undiagnosed LS cases with constitutional *MLH1* promoter methylation²⁰, the incidence of LS is expected to be higher than 2.9% in our population. This result was similar to that found in Finnish and American studies^{3, 21}, and much higher than reported in Mediterranean or Japanese populations where the prevalence was no more than 1%.^{22, 23} However, the overall incidence of dMMR CRCs found in Chinese patients was relatively low compared with western populations. In a population-based study from the Ohio metropolitan area of the United States, the incidence of dMMR was as high as 14%-16%.^{3, 24} These differences might be explained by the variation in epigenetic background among populations. It is well known that sporadic CRCs exhibit dMMR because of somatic inactivation of the *MLH1* gene by promoter methylation in tumours arising along the sessile serrated pathway.²⁵ In western population, the proportion of *MLH1* absence was 70%-80% of all dMMR tumors,^{22, 26} while it accounted for only 47.9% in our Chinese cohort. Importantly, the prevalence of *BRAF*^{V600E} mutation is much higher in CRC tumors with absence of *MLH1* in

western countries than we found in our study population (9.9%). This is probably a function of the younger age of CRC diagnosis in this Chinese cohort, as somatic *MLH1* methylation is much more common in older patients.²⁷

BRAF^{V600E} mutation analysis has been reported to be a valid tool to exclude LS because this mutation is generally absent in patients with LS and is associated with promoter hypermethylation of *MLH1* gene in sporadic CRCs.²⁸ It is recommended to test for the *BRAF*^{V600E} mutation in colorectal tumors with absence of MLH1 protein expression before proceeding to germline sequencing.²⁹ In a population-based screening program for LS in Australia, 154 (75%) *BRAF*^{V600E} mutations were detected in 205 patients with lack of MLH1 protein expression.²⁶ Recent studies have revealed that *BRAF*^{V600E} mutation analysis has a low negative predictive value for *MLH1* promoter methylation, especially in patients aged more than 70 years.³⁰ Using *BRAF* genotyping alone as a prescreening test would increase referral rates for genetic testing over 2-fold compared with *MLH1* methylation testing. Given the low incidence of *BRAF*^{V600E} mutation in our cohort, the test seems less efficient in reducing the number of patients who require germline *MMR* gene sequencing. Based upon our study results, we therefore suggest that Chinese patients with absence of MLH1 on IHC should go directly to germline testing rather than using *BRAF*^{V600E} testing for prescreening.

Another strength of this study lies in the fact that we evaluated the relationship

between the age of onset and prevalence of LS in patients with dMMR tumors. Given that determination of tumor MMR status has advantages in assessing prognosis and directing adjuvant chemotherapy or immunotherapy as well³¹⁻³³, MMR or MSI testing is suggested in all patients with CRC nowadays.^{34,35} Although universal tumor screening provides the highest sensitivity, the low positive predictive value limited its application in large population. We had to balance both sensitivity and positive rate, so that the screening strategy could be more cost-effective. According to our cohort, most patients with LS developed CRC in their 40s or 50s, so the prevalence of LS is very low (2/35, 5.7%) in CRC patients older than 65 years even with dMMR tumors. In this case, universal sequencing for patients with dMMR tumor might not be necessary, and selective sequencing of cases with dMMR and older than 65 years fulfilling the revised Bethesda guidelines result in 8.2% fewer cases requiring germline testing without missing any LS diagnoses. Since germline tests are time consuming and costly, the selective strategy is effective and could be an alternative approach for LS screening.

Interestingly, two common mutations were detected in our cohort. The c.793C>T missense mutation accounted for 16.7% (7/42) of all pathogenic *MLH1* mutation cases and the c.1452_1455 deletion responsible for 16.7% (6/36) of *MSH2* mutation cases identified. Both of these mutations have been reported previously. The former was detected in Taiwanese LS families³⁶ while the latter was found in Hong Kong Chinese.³⁷ Since there were major emigrations in China during the last several

centuries, these two mutations are deemed as founder mutations in the southern Chinese population. Considering that we still have several novel mutations identified in at least two ostensibly unrelated patients, the possibility of additional founder mutations exists and further study is required.

Naturally, this study has limitations. First, direct assay for *MLH1* promoter methylation was not performed, such that the incidence of epigenetic gene inactivation as a cause of dMMR and LS in Chinese CRC patients was unknown. However, the proportion of LS patients with constitutional *MLH1* promoter methylation in other populations is only approximately 1%.²⁰ So this is unlikely to be a large effect in our results. Furthermore, we could not address whether direct methylation testing is a feasible approach for enriching suspected patients for further sequencing than *BRAF* testing. The selective strategy derived from our cohort awaits confirmation in another cohort of patients with CRC. Additional studies will be indispensable to determine its cost-effectiveness. Lastly, further studies of the *MMR* genes with variants of uncertain significance, such as segregation of mutations, functional assays, and somatic sequencing, are important to clarify the pathogenicity of these mutations and guide future surveillance for these families, which will be facilitated by the InSiGHT MMR LOVD and Variant Interpretation Committee, now recognized as the ClinVar Expert Panel for MMR genes.³⁸

To our knowledge, this is one of the most comprehensive studies for LS in CRC

patients. Although the incidence of LS is similar to that of western population, the spectrum of mutations and frequency of sporadic dMMR indicated by MLH1 loss and concurrent *BRAF*^{V600E} mutation is quite different, supporting that LS do has ethnic diversity. The age of onset indicates risk of LS in CRC patients with dMMR tumors. Selective germline sequencing in patients with dMMR tumors diagnosed at age 65 years or younger, and in older patients fulfilling the revised Bethesda guidelines is optimal and could be an alternative approach to universal tumor screening for LS.

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Figure Legends

Figure1 Flow diagram of the screening strategy and main results of the study

CRC, colorectal cancer; IHC, immunohistochemistry; MMR, mismatch repair; dMMR, mismatch repair protein deficiency; pMMR, mismatch repair protein proficiency

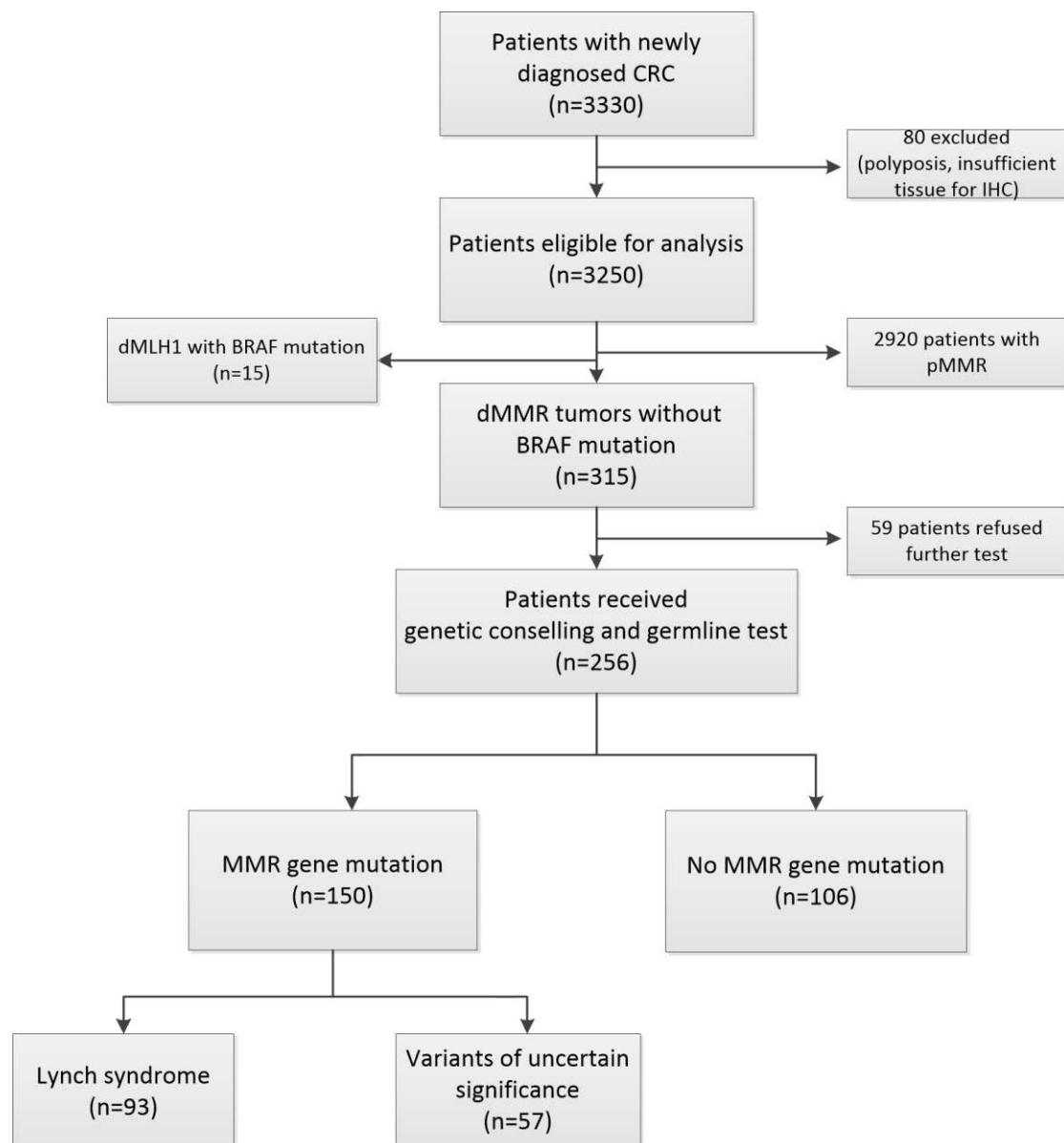
Figure 2 Correlation between age of onset and prevalence of Lynch syndrome in patients with MMR protein deficiency

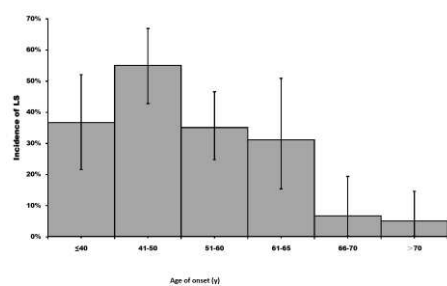
The prevalence of Lynch syndrome (LS) is closely related to age of onset in patients with MMR protein deficiency. The risk of LS is very low (2/35, 5.7%) for patients older than 65 years even with MMR protein deficiency.

Tables

Table1. Demographic and Clinical Characteristics for the 330 patients with MMR protein deficiency

Table2. Performance of different Strategies for the Identification of Patients with Lynch Syndrome





Tables

Table 1. Demographic and Clinical Characteristics for the 330 patients with MMR protein

deficiency

	dMLH1 Alone or With Partner (n=158)	dMSH2 Alone or With Partner (n=98)	Isolate dMSH6 (n=26)	Isolate dPMS2 (n=26)	Other ^a (n=22)	All (n=330)
Age, years						
Mean±SD	53.2±14	51.1±11	55.5±12	55.8±10	53.4±13	53.0±13
Sex, No. (%)						
Male	88 (56)	68 (70)	23 (88)	16 (62)	13 (59)	208 (63)
Female	70 (44)	30 (30)	3 (12)	10 (38)	9 (41)	122 (37)
Tumor stage, No. (%)						
I-III	154 (97)	90 (92)	25 (96)	25 (96)	20 (91)	314 (95)
IV	4 (3)	8 (8)	1 (4)	1 (4)	2 (9)	16 (5)
Multiple LS tumors ^b , No. (%)						
Synchronous	8 (5)	6 (6)	1 (4)	1 (4)	1 (5)	17 (5)
Metachronous	14 (9)	13 (13)	0 (0)	3 (11)	4 (18)	34 (10)
No	136 (86)	79 (81)	25 (96)	22 (85)	17 (77)	279 (85)
Relatives with LS tumors ^c , No. (%)						
Yes	48 (30)	36 (37)	7 (27)	9 (35)	5 (23)	105 (32)
No	110 (70)	62 (63)	19 (73)	17 (65)	17 (77)	225 (68)

^aLoss of 3 MMR proteins or unpaired loss

^bLS tumors: Lynch syndrome related tumors, colorectal, endometrial, ovarian, gastric, hepatobiliary, small bowel, urinary tract, pancreatic, and brain cancer

^cYes, if any first- or second-degree relative with LS tumors.

Table 2. Performance of different Strategies for the Identification of Patients with Lynch Syndrome^a

Case (%)		Diagnostic Yield		Sensitivity		Specificity		PPV		NPV	
Patients requiring MMR testing	Patients requiring germline sequencing	Case/Total No.	% (95% CI)	Case/Total No.	% (95% CI)	Case/Total No.	% (95% CI)	Case/Total No.	% (95% CI)	Case/Total No.	% (95% CI)
Amsterdam criteria II	0 (0)	35 (1.1)	9/3191 (0.3%)	9/93 (9.7%)	3072/3098 (98.9%)	9/35 (25.7%)	3072/3098 (98.9%)	3072/3156 (97.3%)	97.3%	3072/3156 (97.3%)	97.3%
revised Bethesda guidelines	1046 (32.7)	164 (5.1)	76/3191 (2.4%)	76/93 (81.7%)	3010/3098 (97.2%)	76/164 (46.3%)	3010/3098 (97.2%)	3010/3027 (99.4%)	99.4%	3010/3027 (99.4%)	99.4%
Universal screening	3191 (100)	256 (8.0)	93/3191 (2.9%)	93/93 (100%)	2935/3098 (94.7%)	93/256 (36.3%)	2935/3098 (94.7%)	2935/2935 (100%)	100%	2935/2935 (100%)	100%
Selective strategy ^b	3191 (100)	235 (7.4)	93/3191 (2.9%)	93/93 (100%)	2956/3098 (95.4%)	93/235 (39.6%)	2956/3098 (95.4%)	2956/2956 (100%)	100%	2956/2956 (100%)	100%

^a The strategies for Lynch Syndrome screening were evaluated in 3191 patients. Those with MMR protein deficiency but refuse further germline testing (59 patients) were excluded.

^b Selective strategy: Universal tumor MMR testing as the first line screening, then germline sequencing was only performed in patients with MMR protein deficiency who were diagnosed at 65 years or younger, and in older patients fulfilling at least 1 criterion of the revised Bethesda guidelines

Novelty and Impact:

The prevalence of Lynch syndrome (LS) varies significantly in different ethnic populations. In this study, the authors screened more than 3,000 Chinese colorectal cancer patients for mutations associated with LS, including mismatch repair (MMR) and *BRAF*^{V600E} mutations. They found that, while the prevalence of LS in Chinese patients is similar to that of Western populations, the spectrum of mutations is different, including many not previously reported. Older patients had a decreased risk of LS, suggesting that germline sequencing may not be necessary in this population.